UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

SAMILLY EVANGELISTA SOUZA

EFEITO DA ASSOCIAÇÃO DE AMIDO COM SACAROSE NA DESMINERALIZAÇÃO DA DENTINA RADICULAR

EFFECT OF STARCH AND SUCROSE COMBINATION ON ROOT DENTINE DEMINERALIZATION

Piracicaba
2016

SAMILLY EVANGELISTA SOUZA

EFEITO DA ASSOCIAÇÃO DE AMIDO COM SACAROSE NA DESMINERALIZAÇÃO DA DENTINA RADICULAR

EFFECT OF STARCH AND SUCROSE COMBINATION ON ROOT DENTINE DEMINERALIZATION

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Clínica Odontológica, na Área de Prótese Dental.

Thesis presented to Piracicaba Dental School of University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Dental Clinic in Dental Prosthesis area.

Orientador: Prof. Dr. Jaime Aparecido Cury

Coorientadora: Profa. Dra. Altair Antoninha Del Bel Cury

Este exemplar corresponde à versão final da tese defendida pela aluna Samilly Evangelista Souza e orientada pelo Prof. Dr. Jaime Aparecido Cury.

Piracicaba
2016

Agência(s) de fomento e nº(s) de processo(s): CAPES

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Heloisa Maria Ceccotti - CRB 8/6403

So89e

Souza, Samilly Evangelista, 1988-
Efeito da associação de amido com sacarose na desmineralização da dentina radicular / Samilly Evangelista Souza. – Piracicaba, SP : [s.n.], 2016.

Orientador: Jaime Aparecido Cury.
Coorientador: Altair Antonina Del Bel Cury.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Amido. 2. Carboidratos da dieta. 3. Biofilmes. 4. Dentina. I. Cury, Jaime Aparecido,1947-. II. Del Bel Cury, Altair Antoninha,1948-. III. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. IV. Titulo.

Informações para Biblioteca Digital

Título em outro idioma: Effect of starch and sucrose combination on root dentine demineralization
Palavras-chave em inglês:
Starch
Dietary Carbohydrate
Biofilms
Dentin
Área de concentração: Prótese Dental
Titulação: Doutora em Clínica Odontológica
Banca examinadora:
Jaime Aparecido Cury [Orientador]
Carolina Patrícia Aires
Yuri Wanderley Cavalcanti
Antônio Pedro Ricomini Filho
Valentim Adelino Ricardo Barão
Data de defesa: 18-08-2016
Programa de Pós-Graduação: Clínica Odontológica

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 18 de Agosto de 2016, considerou a candidata SAMILLY EVANGELISTA SOUZA aprovada.

PROF. DR. JAIME APARECIDO CURY

PROFª DRª. CAROLINA PATRÍCIA AIRES

PROF. DR. YURI WANDERLEY CAVALCANTI

PROF. DR. ANTÔNIO PEDRO RICOMINI FILHO

PROF. DR. VALENTIM ADELINO RICARDO BARÃO

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

DEDICATÓRIA

Dedico este trabalho aos meus pais, **Iumara e Winder**, que sempre me incentivaram a lutar pelos meus objetivos e ir em busca dos meus sonhos.

AGRADECIMENTOS

Primeiramente agradeço à Deus pelo dom da minha vida e por participar dela todos os dias em tudo que faço, me tornando corajosa e apta a sempre lutar pelos meus sonhos.

À Universidade Estadual de Campinas, na pessoa do Magnifico Reitor, Prof. Dr. José Tadeu Jorge.

À Faculdade de Odontologia de Piracicaba, na pessoa do Diretor Prof. Dr. Guilherme Elias Pessanha Henriques.

À equipe da Coordenadoria de Pós-Graduação da Faculdade de Odontologia de Piracicaba, especialmente a coordenadora Profa. Dra. Cínthia Pereira Machado Tabchoury, a qual considero exemplo de profissional. Obrigada pelos intensos momentos de aprendizagem vivenciados ao longo da pós-graduação.

Ao meu orientador, Prof. Dr. Jaime A. Cury, que com sua sabedoria e vivência me conduziu, fazendo com que eu galgasse com determinação e garra a pós-graduação. Muito obrigada por me fornecer as ferramentas necessárias para o crescimento pessoal e profissional no decorrer deste últimos anos. Será sempre um grande exemplo para mim.

À minha coorientadora, Profa. Dra. Altair A. Del Bel Cury, pela sua disponibilidade e incentivo, os quais foram fundamentais para realizar e prosseguir este estudo. As suas críticas construtivas, as discussões e reflexões foram essenciais ao longo de todo o percurso, não só em relação a este trabalho como também para a minha vida acadêmica.

Ao Prof. Dr. Wander José por ter me aceitado no programa de pós-graduação de clínica odontológica . Obrigada pelo acolhimento e pelos conselhos durante este período.

Aos professores do programa de Pós-Graduação em Clínica Odontológica e em Odontologia, em especial da área de concentração em Prótese Dental e Cariologia, pelos conhecimentos transmitidos. Agradeço especialmente ao Prof. Dr. Valentin Barão, Profa. Dra. Lívia Tenuta, Profa. Dra. Celia Barbosa Rizzati e Profa. Dra. Renata Garcia que de alguma forma

colaboraram para o meu crescimento profissional.

À Sra. Eliete Matim, secretária do Departamento de Prótese e Periodontia da Faculdade de Odontologia de Piracicaba, e as secretárias da coordenadoria geral dos programas de Pós-graduação, meus sinceros agradecimentos pela disponibilidade e gentileza durante esse período.

À todos os funcionários da FOP-UNICAMP, em especial a Sra. Gislaine Piton, técnica do laboratório em Prótese Parcial Removível; Sr. José Alfredo da Silva e Sr. Waldomiro Vieira, os quais muito solícitos, suportaram com delicadeza e boa vontade sempre as minhas ansiedades, recebam o meu carinho e o meu muito obrigada por TUDO!!

À meus pais, pelo melhor que me proporcionaram, sacrificando-se, inúmeras vezes, para que eu pudesse ir em busca dos meus objetivos. Muito obrigada a vocês que me educaram dentro dos valores que me apontam os caminhos a seguir, vencendo os obstáculos com Fé.

À meus avós, em especial a minha avó Zefa e meu avô Othon, que na simplicidade me proporcionaram uma vida cheia de carinho e amor, o que seria de mim sem o apoio de vocês? Obrigada por serem a base da nossa família!

À meus familiares, em nome de tia Diva e a tia Fá, que mesmo longe, sempre me apoiaram e me incentivaram nesta jornada em busca de novos horizontes, muito compreensivas nos meus momentos de ausência. À Rick, para qual quero ser exemplo de motivação e determinação em busca da melhor formação.

Ao meu namorado, só tenho que dizer muito obrigada por toda força durante esse período. Obrigada por sempre estar ouvinte e atento a minhas inquietações, dúvidas, e algumas vezes, desânimos. Obrigada pela confiança e pela valorização ao meu trabalho, pelo incentivo e por sempre ter entendido as ausências e a minha dedicação a pós-graduação. Sempre com as palavras certas, no momento certo, para acalmar meu coração!

Às minhas amigas, em especial Luiza Figueiredo, Bruna Ximenes, Kelly Andrade e Simone Valenga, a amizade de vocês foi fundamental na minha vida em Piracicaba, e vou levar comigo pra sempre. À Aline Castro e Maíra Barbosa que, mesmo com a distância, foram

muito presentes durante essa etapa de minha vida. À Inara Pereira, que apesar do pouco tempo de convívio, me apoio em vários momentos difíceis.

Um agradecimento especial a Prof. Dr. Yuri Cavalcanti, Prof. Dr. Antônio Pedro Ricomini e a amiga Aline Sampaio pelos trabalhos desenvolvidos juntos, pelos estudos e experimentos realizados incessantemente durante os finais de semana, e pela grande amizade que fizemos!! Essa trajetória foi muito menos árdua com a companhia e a parceria de vocês.

À todos os meus colegas de pós-graduação da FOP-UNICAMP, por dividirem comigo cada momento de aprendizagem. Agradeço especialmente a Adaias Matos, André Gazzeta, Barbara Oliveira, Bruna Alfenas, Camila Lima, Carolina Veloso, Diego Nóbrega, Dimorvan Bordin, Edmara Bergamo, Emanuele Vieira, Giancarlo De La Torre, João Gabriel, Juliana Botelho, Lívia Foster, Louise Donatelas, Marco Aurélio, Rafael Soares, Priscilla Lazari e Victor Munõz pelo apoio, confiança e partilha em todos os momentos dessa grande conquista, o meu muito obrigada e o desejo de que Deus olhe para cada um, ajudando-os a vencer.

Meu muito obrigada à todos que, direta ou indiretamente, contribuíram para minha formação e crescimento durante essa minha caminhada.

RESUMO

Sacarose é o carboidrato mais cariogênico da dieta, mas na presença do amido esta cariogenicidade pode ser potencializada provocando maior desmineralização na estrutura dental. Entretanto, resultados conflitantes têm sido encontrados em relação ao potencial cariogênico da associação amido+sacarose para dentina, o que pode estar relacionado com diferenças nos modelos de estudo usados. Estudos *in vitro* que avaliaram a cariogenicidade de produtos amiláceos para a dentina foram realizados com modelo de biofilme de *S. mutans*, não composto por bactérias implicadas com o metabolismo direto ou indireto do amido. No presente estudo, nós expandimos o conhecimento sobre este assunto utilizando um modelo de biofilme composto por *S. mutans* (*Sm*), *S. gordonii* (*Sg*) e *A. naeslundii* (*An*). *Sm* é a bactéria bucal mais cariogênica, o *An* possui atividade amilolítica e *Sg* por ser colonizador inicial que expressa uma proteína que liga α–amilase, enzima salivar indispensável para o metabolismo do amido na cavidade bucal. Assim, o objetivo deste trabalho foi avaliar o efeito da associação amido+sacarose na desmineralização da dentina radicular, utilizando modelo de biofilme tri-espécie, contemplando a atividade da amilase salivar. Foi realizado estudo *in* vitro, cego, conduzido em 3 experimentos independentes com n=6 (*n* final=18). Biofilmes de *Sm*+*Sg*+*An* foram crescidos sobre blocos de dentina radicular bovina (7x4x1 mm), cujas durezas de superfície foram previamente determinadas. Biofilme foram crescidos em meio de cultura ultrafiltrado à base de triptona e extrato de levedura contendo glicose 0,1 mM, os quais foram expostos a 4 grupos de tratamentos: NaCl 0,9% (controle negativo), amido 1%, sacarose 10% ou amido 1% + sacarose 10%. Os tratamentos foram realizados por 3 min, 8 vezes/dia, durante 3 dias. Para simular o efeito da α–amilase, os biofilmes foram tratados (1 min) com saliva humana antes de cada exposição aos tratamentos, a qual também foi adicionada ao meio de cultura. Para avaliar a acidogenicidade dos biofilmes, o pH do meio de cultura foi determinado 2x/dia, após as 8 exposições diárias aos tratamentos e após o período noturno de jejum. Após 96 h, os biofilmes foram coletados para análises de viabilidade bacteriana, biomassa e quantidade de polissacarídeos. A desmineralização dentinária foi avaliada por porcentagem de perda de dureza de superfície (%PDS). A bioarquitetura do biofilme foi analisada qualitativamente por microscopia confocal a laser (CLSM). Os dados quantitativos foram analisados por ANOVA seguido por teste de Tukey, com nível de significância de 5%. Biofilmes expostos à amido+sacarose mostraram ser mais acidogênicos ($p = 0,007$) em comparação com sacarose, provocando após as 8 exposições um menor valor de pH do meio de cultura ($4,89 \pm 0,29$ vs $5,19 \pm 0,32$). O tratamento com amido+sacarose

provocou maior %PDS (53,2 ± 7,0) quando comparado com sacarose (43,2 ± 8,7). Os biofilmes tratados com amido+sacarose não diferiram significativamente do grupo sacarose em relação à biomassa, viabilidade bacteriana e quantidade de polissacarídeos ($p > 0,05$). Análise por CLSM confirma polissacarídeos extracelulares são encontrados somente na matriz dos biofilmes exposto a amido+sacarose e sacarose. Os resultados obtidos mostram que a associação amido+sacarose é mais cariogênica para a dentina radicular que o efeito da sacarose.

Palavras-chave: Amido. Carboidratos da dieta. Biofilmes. Dentina

ABSTRACT

Sucrose is the most cariogenic dietary carbohydrate but in the presence of starch this cariogenicity can be increased. However, conflicting results have been found about the cariogenic potential of starch + sucrose combination to dentin, which may be related to the differences in the models of evaluation used. *In vitro studies* that evaluated the cariogenic potential of starchy products to dentine were made with *S. mutans* biofilm model, not composed of bacteria involved with the direct or indirect metabolism of starch. In this study, we have expanded the knowledge on this subject using a biofilm model composed of *Sm* is the most cariogenic oral bacteria, *An* has amylolytic activity and *Sg* is an initial colonizer that expresses a binding protein to α-amylase, which are essential bacteria for the starch metabolism in the oral cavity. The objective of this study was to evaluate the effect of the starch + sucrose combination in root dentin using a tri-species biofilm model, contemplating the salivary amylase activity. An in vitro and blind study was conducted in three independent experiments with n=6 (*n* total = 18). Biofilm *Sm*+*Sg*+*An* were grown on bovine root dentin blocks (7x4x1 mm), which surface hardnesses were previously determined. The biofilms were grown in ultrafiltered tryptone-yeast extract broth containing 0.1 mM glucose and they were exposed to 4 treatment groups: 0.9% NaCl, 1% starch, 10% sucrose or 1% starch + 10% sucrose. The treatments were carried out for 3 min, 8 times per day for 3 days. To simulate the effect of α–amylase, biofilms were treated (1 min) with human saliva stimulated before each treatment exposure and salivary amylase was also added into the culture medium. To evaluate the biofilms acidogenicity, the culture pH of culture medium was determined 2x/day, after the 8 treatments exposure and after an overnight period. After 96 h, the biofilms were collected for analyses of bacterial viability, biomass and quantity of polysaccharides. The dentine demineralization was assessed by surface hardness loss (%SHL). The biofilm bio-architecture was qualitatively analyzed by confocal microscopy laser scanning (CLSM). The quantitative data were analyzed by ANOVA followed by Tukey's test at 5% significance level. Biofilms treated with starch + sucrose were more acidogenic ($p = 0.007$) than those exposed to sucrose alone, showing after the 8 treatments exposure a lower culture medium pH (4.89 ± 0.29 *vs.* 5.19 ± 0.32). Treatment with starch + sucrose combination provoked greater demineralization (%SHL= 53.2 ± 7.0) than sucrose group (43.2 ± 8.7). Biofilms treated with starch + sucrose did not differ statistically from the sucrose group regarding biomass, bacterial viability and the amount of polysaccharides ($p > 0.05$). The CLSM analysis confirms that extracellular polysaccharides are found only in matrix of biofilms formed in the presence

of starch + sucrose combination or sucrose. The findings show additional experimental evidence that starch + sucrose combination is more cariogenic for root dentine than the isolated effect of sucrose.

Keywords: Starch. Dietary Carbohydrates. Biofilms. Dentine

SUMÁRIO

1 INTRODUÇÃO

A cárie dentária é caracterizada pela perda progressiva de mineral da estrutura dental pelos ácidos produzidos por bactérias do biofilme dentário quando esses são regularmente expostos à carboidratos da dieta (Fejerskov, 2004). A doença cárie não afeta apenas as crianças e adultos, mas pode ser considerada uma preocupação de saúde bucal para idosos devido não só ao aumento da expectativa de vida como a uma série de fatores (Saunders and Meyerowitz, 2005), como a presença de recessão gengival, falta de higiene dental, falta de destreza manual, entre outros, os quais podem elevar a suscetibilidade dos idosos em relação a cárie radicular (Gupta et al., 2006). Além disso, há um número substancial de pessoas nessa faixa etária cuja capacidade de mastigar alimentos é comprometida devido à ausência parcial de dentes (McKenna et al., 2012). O desconforto gerado durante a mastigação faz com que esses indivíduos evitem consumir frutas e legumes (Wall e Steele, 2004), preferindo alimentos mais fáceis de serem mastigados, como os produtos amiláceos, o que pode aumentar o risco de cárie radicular.

A dieta com carboidratos fermentáveis atua como fator determinante para o desenvolvimento de lesões de cárie por essa ser uma doença biofilme-açúcar dependente (Fejerskov, 2004). Sacarose é o mais cariogênico carboidrato da dieta (Paes Leme et al., 2006), pois além de ser facilmente fermentada à ácidos (Newbrun, 1967), ele é o único açúcar da dieta que é substrato bacteriano para a síntese de polissacarídeos extracelulares (PECs) solúveis e insolúveis (Rolla et al., 1985; Cury et al., 2000). Entre esses PECs, os insolúveis estão relacionados à organização estrutural do biofilme, aumentando a sua porosidade (Dibdin e Shellis, 1988), o que facilita a difusão dos carboidratos da dieta pelo biofilme até camadas próximas da superfície dental. Essa difusão facilitada provocará maiores quedas de pH no biofilme, o qual abaixo de 6,5 já é crítico para a dissolução da dentina (Hoppenbrouwers et al., 1987).

Por outro lado, alguns carboidratos da dieta, como por exemplo o amido, são considerados moderadamente ou não cariogênicos (Lingstrom et al., 1994; Aires et al., 2008). Ao contrário da sacarose, o amido não é facilmente fermentado pelas bactérias bucais, pois: 1) sua difusão para o interior do biofilme é dificultada devido ao seu alto peso molecular(Thurnheer et al., 2003); 2) ele tem que ser primeiro quebrado pela amilase salivar para que os produtos da hidrólise sejam fermentados pelas bactérias (Fiehn et al., 1983). Além disso, amido sozinho não é

substrato para a síntese de PECs (Ribeiro et al., 2005). Porém, quando dissolvido (gelatinizado), o amido é rapidamente degradado pela amilase salivar e por enzimas bacterianas, gerando maltose e amilodextrinas como subprodutos (Tester et al., 2006, Fiehn et al., 1983). Enquanto maltose é facilmente fermentada a ácidos, as amilodextrinas podem servir como aceptores para a síntese PECs. PECs sintetizados na presença do amido + sacarose apresentam estruturas diferentes dos sintetizados a partir apenas da sacarose (Bowen e Koo, 2011). Assim, o biofilme formado na presença do amido + sacarose pode ter propriedades mais cariogênicas quando comparado à sacarose pelo fato de apresentar polissacarídeos com maior porcentagem de cadeias ramificadas, o que tem sido estudado experimentalmente.

Assim, Vacca-Smith et al. (1996) mostraram que polissacarídeos sintetizados por glicosiltransferases (GTF), tendo como substratos sacarose e hidrolisados de amido, possuem estruturas químicas diferentes daqueles produzidos apenas com sacarose. Esse resultado suscitou o desenvolvimento de pesquisas para comprovar se estes polissacarídeos diferenciados poderiam contribuir para a cariogenicidade do biofilme, aumentando a desmineralização do esmalte-dentina. Ribeiro et al. (2005) mostraram, em estudo in situ, que biofilmes expostos 8x/dia à associação amido + sacarose provocaram maior desmineralização do esmalte de dentes decíduos que sacarose isoladamente. Entretanto, eles não encontraram diferença na concentração de polissacarídeos insolúveis nos biofilmes formados na presença dessa combinação quando comparado a sacarose, porém a estrutura desses polissacarídeos não foi determinada. Em outro estudo in situ, Aires et al. (2008) mostraram que a combinação amido + sacarose mostrou tendência de provocar maior desmineralização da dentina radicular que sacarose, entretanto a diferença não atingiu nível de significância estatística. Com relação à composição dos biofilmes em termos de concentração de PECs os resultados confirmaram os encontrados por Ribeiro et al. (2005).

Em relação às características estruturais, Duarte et al. (2008) em um estudo in vitro mostraram que biofilmes de *S. mutans* formados sobre discos de hidroxiapatita (HA) e expostos continuamente à associação amido + sacarose apresentaram maior quantidade de polissacarídeos extracelulares insolúveis que aqueles expostos à sacarose. Esses autores também mostraram que a queda de pH foi maior quando da exposição a amido + sacarose. Nessas mesmas condições, Klein et al. (2009) demonstraram que os biofilmes formados pela exposição à amido + sacarose possuíam a matriz extracelular estruturalmente diferente daqueles formados na presença de

sacarose, sugerindo maior quantidade de PECs insolúveis altamente ramificados. Entretanto, os resultados desses estudos (Duarte et al. 2008; Klein et al. 2009) não permitem concluir que o amido aumenta a cariogenicidade da sacarose, pois não foi avaliada a desmineralização provocada no substrato mineral (HA). Em acréscimo, os biofilmes foram expostos constantemente aos carboidratos, não sendo simulada a exposição intermitente que ocorre na boca pela dieta.

Em contrapartida, Thurnheer et al. (2008) avaliaram in vitro se a associação amido + sacarose provocaria maior desmineralização do esmalte que o efeito isolado da sacarose, usando um modelo multi-espécies de biofilme formado sobre discos de esmalte bovino. A desmineralização foi avaliada por QLF (fluorescência quantitativa induzida por luz). Os autores concluíram que a combinação amido + sacarose não potencializa o poder cariogênico da sacarose. Entretanto, como os biofilmes foram continuamente expostos aos carboidratos, o pH foi mantido em valores extremamente baixos não permitindo diferenciar o poder cariogênico dos carboidratos testados.

Recentemente, Botelho et al. (2016) mostraram que a combinação amido + sacarose provocou maior desmineralização não só do esmalte como da dentina radicular que o efeito isolado da sacarose. Nesse estudo in vitro, não apenas a exposição intermitente a açucares foi simulada como também a degradação do amido pela amilase salivar foi contemplada pelo uso de saliva humana. Entretanto, o modelo de biofilme usado foi composto apenas por *S. mutans*, e embora ele tenha sido validado (Ccahuana-Vásquez et al, 2010) e tenha já sido usado para avaliar outros produtos da dieta (Muñoz-Sandoval et al., 2012; Giacaman et al., 2012), outras bactérias bucais tem papel importante na metabolização do amido. Assim, embora *S. mutans* seja considerada a bactéria bucal mais cariogênica, ela não tem capacidade amilolítica e não potencializa o efeito da amilase salivar, ficando em dúvida se esses resultados seriam confirmados usando um modelo de biofilme mais apropriado.

Nesse sentido, objetivando avaliar a cariogenicidade de produtos da dieta, particularmente os amiláceos, Cavalcanti et al. (2014) desenvolveram um modelo de biofilme composto de *S. mutans* (Sm), *A. naeslundii* (An) e *S. gordonii* (Sg). A presença de *Sm* foi justificada por ser esta considerada a mais cariogênica das bactérias bucais pois além de ser acidogênica e acidúrica, ela sintetizar PECs a partir de sacarose (Paes Leme et al., 2006). *An* desempenha papel importante no metabolismo do amido, pois *An* possui capacidade de quebrar

as ligações glicosídicas α–1,4 do amido devido a presença de uma enzima α-amilase (Glor et al., 1988). *Sg* por meio da proteína AbpA proteína serve de sítio de ligação para a amilase salivar (Nikitkova et al., 2013), contribuindo indiretamente para a metabolização do amido pelas bactérias do biofilme. Entretanto, para o desenvolvimento desse modelo, sacarose foi o carboidrato usado, não sendo testados produtos amiláceos.

Logo, tendo em vista as limitações dos estudos já realizados e que a dúvida maior sobre a cariogenicidade da associação amido + sacarose é com relação a dentina, o objetivo do presente trabalho foi avaliar se essa combinação provoca maior desmineralização na dentina que o efeito isolado de sacarose, usando para tal um modelo de biofilme mais adequado para avaliar o potencial cariogênico de produtos amiláceos.

2 ARTIGO

STARCH INCREASES ROOT DENTINE DEMINERALIZATION PROVOKED BY SUCROSE

Artigo submetido ao periódico Caries Research (Anexo 1)

Samilly Evangelista **Souza**[1], Aline Araújo **Sampaio**[1], Altair Antoninha **Del Bel Cury**[1], Yuri Wanderley **Cavalcanti**[2], Antônio Pedro **Ricomini Filho**[1], Jaime Aparecido **Cury**[1]

[1]Piracicaba Dental School, UNICAMP, Piracicaba, SP, Brazil
[2]Federal University of Paraiba, João Pessoa, PB, Brazil

Short Title: Dentine demineralization by starch/sucrose

Keywords: Starch. Sucrose. Biofilm. Dentine. Demineralization

Corresponding Author:
Prof. Jaime A. Cury
Piracicaba Dental School, UNICAMP
CP 52
13414-903 Piracicaba, SP, Brazil
Tel.: 55-19-21065303
E-mail: jcury@unicamp.br

Declaration of Interests

There are no conflicts of interest with respect to the authorship and/or publication of this article.

Abstract

Most studies evaluating whether starch increases the cariogenic potential of sucrose have used the monospecies *Streptococcus mutans* biofilm model. To extend the knowledge about this subject, we used a biofilm model composed of *S. mutans, S. gordonii,* and *Actinomyces naeslundii*, which was originally developed to evaluate the cariogenicity of starchy foods. Biofilms (n = 18) were formed on root dentine slabs under exposure 8x/day to one of the following treatments: 0.9% NaCl, 1% starch, 10% sucrose, or a combination of 1% starch and 10% sucrose. Before each treatment, biofilms were pre-treated with human whole saliva for 1 min. The pH of the culture medium was measured daily as an indicator of biofilm acidogenicity. After 96 h of growth, biofilms were collected and the biomass, bacteria viability, and polysaccharides were analyzed. Dentine demineralization was assessed by surface hardness loss (%SHL). Biofilm bio-architecture was analyzed using confocal laser scanning microscopy. Treatment with starch and sucrose combination provoked higher ($p = 0.01$) dentine demineralization than sucrose alone (%SHL = 53.2 ± 7.0 *vs.* 43.2 ± 8.7). This was supported by lower pH values ($p = 0.007$) of the culture medium after daily exposure to starch and sucrose combination compared with sucrose (4.89 ± 0.29 *vs.* 5.19 ± 0.32). Microbiological and biochemical findings did not differ between biofilms treated with combination of starch and sucrose and sucrose alone ($p > 0.05$). The present findings from this biofilm model give support that a combination of starch and sucrose is more cariogenic than sucrose alone.

Introduction

Starch alone is considered non-cariogenic for enamel and slightly cariogenic for dentine [Sheiham et al., 2001]. In contrast to sucrose, starch is not easily metabolized by oral bacteria because: (1) it has a high molecular weight so cannot easily diffuse through biofilm [Thurnheer et al., 2003]; (2) it must be hydrolyzed before its fermentation by dental biofilm bacteria [Fiehn and Moe, 1983]; and (3) extracellular polysaccharides (EPS) are not produced from starch [Ribeiro et al., 2005]. Although starch is often consumed simultaneously with sucrose [Lingström et al., 2000], it remains unclear whether starch increases the cariogenic potential of sucrose.

In natura starch is not easily fermented to acids in the oral cavity. However, industrialized soluble starch (gelatinized) is easily degraded into maltose and amylodextrin by salivary amylase [Jacobsen et al., 1972; Fiehn and Moe, 1983] and bacterial enzymes [Birkhed and Skude, 1978; Mormann and Muhlemann, 1981]. Further, maltose is easily fermented to acids, and amylodextrin is an acceptor for EPS synthesis [Bowen and Koo, 2011]. EPS synthesized in the simultaneous presence of starch and sucrose has a distinct chemical structure than those synthetized from sucrose alone [Vacca-Smith et al., 1996]. Thus, biofilm formed in the presence of starch and sucrose may be more cariogenic compared with sucrose alone, due to changes in matrix organization [Xiao and Koo, 2010]. This has been investigated *in vitro* and *in situ*.

In vitro studies have suggested that biofilms formed by *S. mutans* on hydroxyapatite disks (HA) in the continuous presence of starch and sucrose have unique biochemical properties [Duarte et al., 2008; Klein et al., 2009; Xiao and Koo, 2010]. Duarte et al. [2008] showed that these biofilms produce more EPS and lower pH culture medium than those exposed to sucrose. Furthermore, the EPS formed are more branched [Klein et al., 2009]. However, these studies did not evaluate whether biofilms formed in presence of starch and sucrose provoked greater HA demineralization than sucrose alone.

Thus, Thurnheer et al. [2008] and Botelho et al. [2016] evaluated the effect of starch and sucrose on the demineralization of dental substrates. Thurnheer et al. [2008] used a multi-species biofilm formed on bovine enamel and concluded that starch and sucrose did not increase the cariogenic effect of sucrose. However, in this study, the biofilms were continuously exposed to carbohydrates, which do not simulate the intermittent exposure to food in real life. Consequently, the low pHs reached do not allow differentiate the cariogenic potential of the treatments. Recently, Botelho et al. [2016], using a monospecies *S. mutans* biofilm model that mimics the

intermittent exposure to carbohydrates that occurs in oral cavity [Ccahuana-Vásquez and Cury, 2010], showed that starch and sucrose increased enamel and dentine demineralization compared with sucrose alone. Regarding *in situ* studies, whilst Ribeiro et al. [2005] showed that biofilms exposed eight times daily to starch and sucrose caused greater demineralization of deciduous enamel than sucrose alone, Aires et al. [2008] were not able to show the same effect for root dentine.

Therefore, while the effect of starch and sucrose combination for enamel is supported by *in vitro* and *in situ* studies, the findings for dentine remain inconclusive. Thus, the aim of the present study was to clarify whether starch and sucrose together are more cariogenic to root dentine than sucrose alone. For this purpose, we used a biofilm model that was developed to evaluate the cariogenic potential of starchy dietary products [Cavalcanti et al., 2014]. This biofilm is composed of *S. mutans* (the most cariogenic bacteria), *A. naeslundii,* (which has amylolytic activity) [Glor et al., 1988] and *S. gordonii,* (which binds salivary amylase) [Nikitkova et al., 2013].

Materials and Methods

Experimental design and ethical issues

An *in vitro*, randomized, and blinded study was conducted. Three-species biofilms composed of *Streptococcus mutans* UA159, *Streptococcus gordonii* ATCC 35105, and *Actinomyces naeslundii* ATCC 12104 were formed on saliva-coated bovine dentine slabs. Biofilms were formed according to the model described by Cavalcanti et al. [2014], except for pre-treatment with human whole saliva before each carbohydrate exposure that was made in this study. Biofilms were grown in ultrafiltered, buffered tryptone-yeast extract broth (UTYEB), and exposed 8 times/day to one of the following treatments: 0.9% NaCl, 1% starch, 10% sucrose, or 1% starch plus 10% sucrose (starch+sucrose). To simulate the effect of salivary amylase, saliva was added to the culture medium, and the biofilms were also pre-treated with saliva before treatment. Culture medium was changed twice daily, at the beginning and at the end of treatment. The pH was measured as an indicator of biofilm acidogenicity. After 96 h, the biomass (biofilm wet weight), viable bacteria count (colony forming units, CFU/mg wet weight), and amount of polysaccharides/biofilm were measured. Dentine demineralization was calculated by the percentage surface hardness loss (%SHL). For statistical analysis, each biofilm/slab was

considered as an experimental unit. Each experiment was repeated three times, with six replicates per group (n = 18/group). The bio-architecture of biofilms was determined using confocal laser scanning microscopy from an independent experiment (n = 3/group). This study was approved by the Research and Ethics Committee of Piracicaba Dental School (Protocol No. 035/2012).

Preparation of root dentine slabs

Root dentin slabs (7 × 4 × 1 mm) were obtained from bovine incisors [Hara et al., 2003]. Baseline surface hardness (SH) was determined by making three indentations with 5 g load for 5 s, 100 μm apart using a Knoop micro-hardness tester coupled to FM-ARS 900 software (Future-Tech Corp., Kawasaki, Japan). Slabs with a mean (± SD) SH of 37.10 ± 7.42 kg/mm^2 were selected and randomly allocated to one of the treatment groups. Slabs were sterilized with ethylene oxide.

Saliva collection and salivary pellicle formation

Whole saliva was collected from three healthy volunteers (24–27 years old) who did not use mouthwash, antimicrobials, or any other drug that affects the saliva, for three months before the study. All participants gave written informed consent to donate saliva for this study.

Stimulated whole saliva was used to form salivary pellicle on dentine slabs, to pre-treat the biofilms before the treatments, and as ingredient of the culture medium. Saliva was collected and prepared as described by Botelho et al. [2016]. Salivary amylase activity was assessed using the lugol test. Salivary pellicles were formed on dentine slabs according to Koo et al. [2000].

Biofilm growth

Biofilms were developed as described by Cavalcanti et al. [2014], with a modified pre-treatment of the biofilms with saliva and inclusion of saliva in the culture medium to simulate the effect of salivary amylase [Botelho et al., 2016]. In addition, the concentration of phosphate buffer in the culture medium was increased 10 times during the 6 h bacterial adhesion phase to avoid a pH drop and dentine demineralization before biofilm formation.

Treatments

Carbohydrates used and solution concentrations were the same as described by Botelho et

al. [2016]. Starch and sucrose solutions were prepared from Sigma Chemical Co., St. Louis, USA (soluble starch; 80% amylopectin and 20% amylose) and Merck Millipore, Darmstadt, Germany, respectively. To prepare 1% starch and 1% starch + 10% sucrose, the suspensions were boiled until total dissolution. All solutions were prepared in 0.9% NaCl (because salivary amylase is activated by chloride ions), autoclaved, and stored at room temperature.

Biofilms grown on dentine surfaces were treated 8 times/day for 3 min at defined times (8:00, 9:30, 11:00, 12:00, 13:30, 15:00, 16:00, and 17:30 h). Before treatment, the slabs were pre-treated for 1 min with 10% (v/v) saliva [Botelho et al., 2016]. This treatment was repeated during the 3 days of biofilm growth.

Biofilm acidogenicity

The culture medium was changed twice daily, before (after overnight biofilm starvation), and after the 8 treatments made during the day. The pH of the medium was measured as an indicator of biofilm acidogenicity, using a pH electrode (Orion 8102BNUWP ROSS Ultra, Thermo Scientific, St Louis, USA) connected to a pH meter (Orion 720A, Thermo Scientific, St Louis, USA) that was previously calibrated (buffers 4.0 and 7.0).

Biofilm collection and analyses

After 96 h, each dentine slab with biofilm were washed three times in 0.9% NaCl to remove non-attached cells and transferred to microcentrifuge tubes containing 1 mL of 0.9% NaCl. The tubes were sonicated at 7W for 30 sec (Branson Sonifier, 150, Danbury, USA) to remove the biofilm [Ccahuana-Vásquez and Cury, 2010]. The slabs were separated and stored for dentine demineralization analysis (% SHL). Aliquots of biofilm suspension were used to determine viable bacteria, biomass, and the amount of extracellular and intracellular polysaccharides.

To determine bacterial viability, 100 µL of biofilm suspension was ten-fold serially diluted in 0.9% NaCl. Three 20 µL aliquots of each dilution were plated on blood agar for total bacteria quantification. The plates were incubated for 48 h at 37 °C, in 10% CO_2. Colony forming units (CFU) were counted and the results expressed as $\log_{10}$ CFU /biofilm.

Biofilm wet weigh (biomass) was calculated from an aliquot of 400 μL of the suspension. This aliquot was transferred to pre-weighed microcentrifuge tubes and centrifuged at 10,000 *g* for 5 min at 4°C. The supernatant was carefully removed and the tube was weighed again.

Extracellular soluble (SEPS) and insoluble polysaccharides (IEPS), and intracellular polysaccharides (IPS) were extracted and determined from a 400 μL aliquot of the suspension, as described by Aires et al. [2008].

Dentine demineralization assessment (%SHL)

SH of dentine slabs was again determined as described above. Three indentations were made, 100 μm apart and 100 μm from three baseline measurements. The percentage of SH loss (%SHL) was calculated [Cury et al., 2000] and used as indicator of dentine demineralization [Vale et al., 2011].

Statistical analysis

Homogeneity of variance and normal distribution of errors were evaluated by the Kolmogorov-Smirnov test. The data of viable bacteria were logarithmically transformed (log_{10}) to attend these requirements. Treatment effects were analyzed by one-way ANOVA followed by Tukey's HSD test. SPSS Statistic software version 20.0 (IBM, Chicago, Illinois) was used for the analysis, and the significance level was set at 5%.

Confocal laser scanning microscopy (CLSM)

Biofilm architecture and organization was observed by CLSM in an independent experiment (n = 3/group) under the same conditions described above. Alexa Fluor 647-dextran conjugate (molecular weight, 10,000; excitation 650 nm/ emission 668 nm; Thermo Scientific, USA) was added to the culture medium [Xiao and Koo, 2010] to visualize EPS in the biofilm matrix. After 4 days of biofilm growth, bacterial cells were stained with SYTO-9 green fluorescent nucleic acid (excitation 485 nm/ emission 498 nm; Thermo Scientific, USA) as describe by Klein et al. [2009]. The images were obtained in a DMI 6000 CS inverted microscope coupled to TCS SP5 computer-operated confocal laser scanning system (Leica Microsystems CMS, Mannheim, Germany), using a 40× oil immersion objective (numeric aperture 1.25). A series of images were obtained from the base of the biofilm to the top, at an

interval of 1 μm in the Z-axis. Three-dimensional reconstructions were made using Image J software [Hartig, 2013] to visualize biofilm organization after the different treatments.

Results

pH changes in the culture medium immediately after daily treatments (32, 56, and 80 h) and night starvation periods (48, 72, and 96 h) are shown in Figure 1. The pH decrease was highest in the starch+sucrose group after 32, 56, and 80 h of biofilm growth ($p < 0.05$). pH changes in the sucrose and starch+sucrose groups were different from the NaCl (negative control) group at 48, 72, and 96 h ($p < 0.05$), but no differences were observed between them. The starch group did not differ from the NaCl group at any time point.

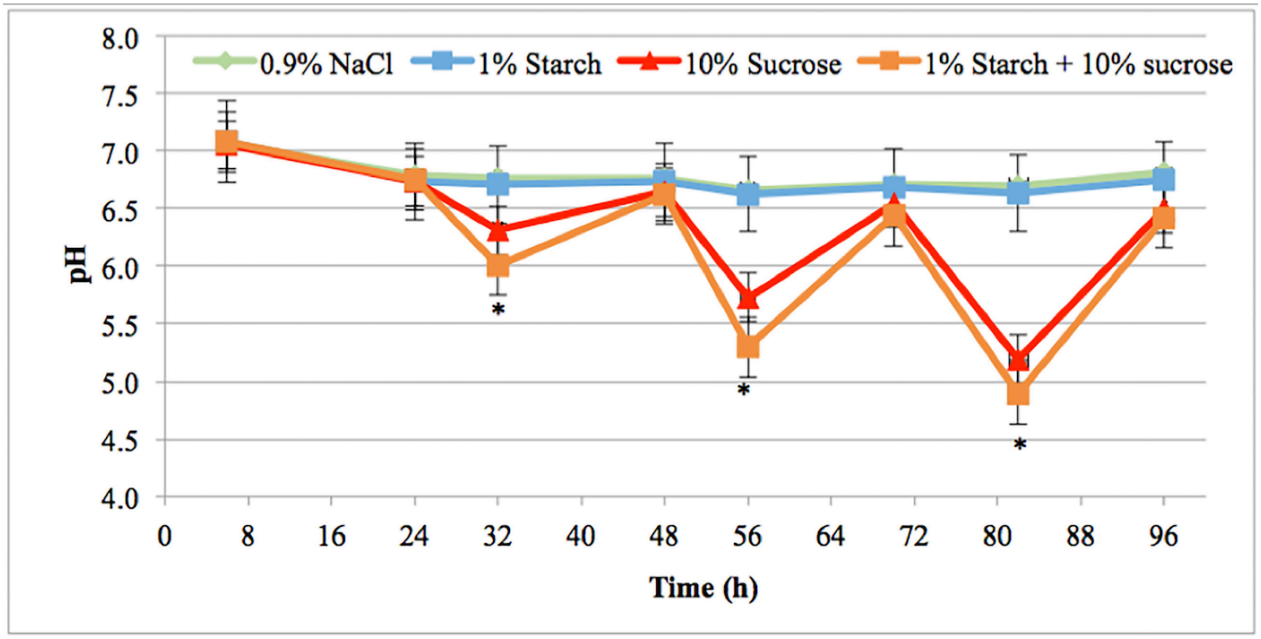

Figure 1. pH values (mean ± SD; n = 18) of the culture medium according to the treatments and time (h) of biofilm growth. *Differences observed between starch+sucrose and sucrose groups ($p < 0.05$).

Biofilms treated with sucrose and starch+sucrose had more viable bacteria, wet weight and polysaccharides (Table 1) than those exposed to starch or to 0.9% NaCl ($p < 0.05$). However, these variables did not differ between the sucrose and starch+sucrose groups ($p > 0.05$). Similarly, starch treatment did not differ significantly from the negative control group ($p > 0.05$).

Table 1. Total viable bacteria, biofilm wet weight (biomass), and polysaccharides in the biofilm formed on each dentine slab, according to the treatments. (Mean ± SD, n = 18).

Treatments	Viable bacteria (CFU log_{10}/biofilm)	Biomass (mg/biofilm)	Polysaccharides (μg/biofilm)		
			SEPS	IEPS	IPS
0.9% NaCl	8.2 ± 0.4[a]	5.0 ± 3.1[a]	7.3 ± 2.3[a]	13.5 ± 3.5[a]	9.9 ± 6.3[a]
1% Starch	8.3 ± 0.5[a]	5.7 ± 3.0[a]	9.0 ± 4.4[a]	15.2 ± 4.7[a]	9.8 ± 5.1[a]
10% Sucrose	9.1 ± 0.3[b]	13.0 ± 7[b]	20.4 ± 6.6[b]	156.0 ± 65.1[b]	46.3 ± 22.8[b]
1%Starch + 10% Sucrose	9.3 ± 0.3[b]	13.3 ± 7[b]	22.7 ± 9.7[b]	171.6 ± 65.1[b]	53.0 ± 23.2[b]

SEPS = soluble extracellular polysaccharides; IEPS = insoluble extracellular polysaccharides; IPS = intracellular polysaccharides. Different letters (within columns) indicate statistically significant differences between treatments ($p < 0.05$)

The biofilms exposed to sucrose and starch+sucrose had a well-structured biofilm compared with those exposed to starch or to NaCl (Figure 2). More microcolonies were detected in the biofilms formed on dentine in the presence of starch than NaCl. EPS (shown in red) were clearly visible in biofilms formed under exposure to sucrose or to starch+sucrose, but not in the other groups.

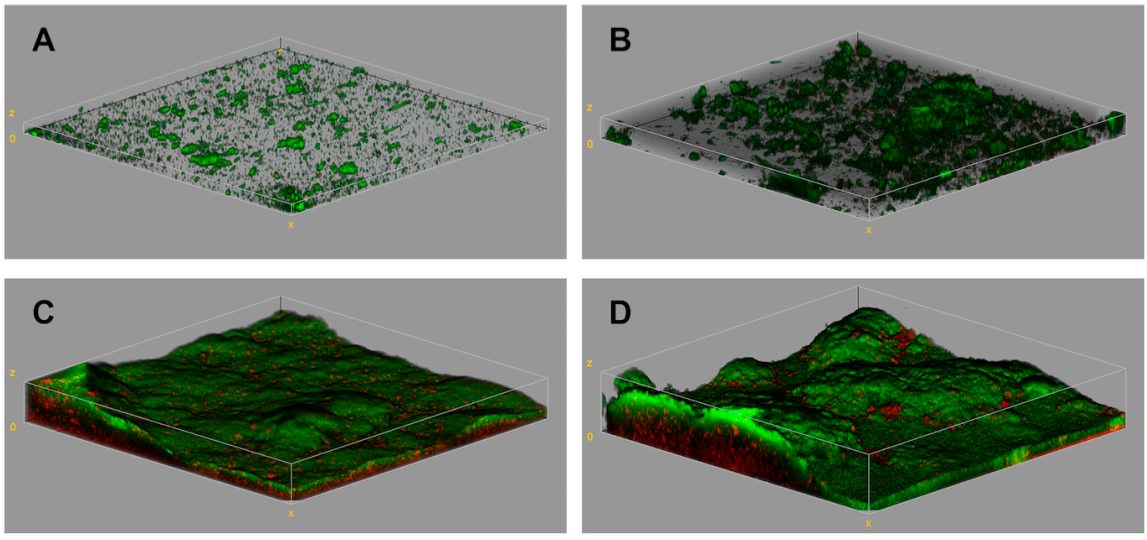

Figure 2. Confocal laser scanning microscopy images of the biofilms according to the treatments. A - 0.9% NaCl; B - 1% starch; C - 10% sucrose; D - 1% starch + 10% sucrose. Green staining: bacterial cells stained with Syto-9. Red staining: extracellular polysaccharides labeled with Alex Fluor 647–dextran conjugate.

Dentine demineralization (%SHL) (Figure 3) was highest in dentine slabs from the starch+sucrose group ($p < 0.05$). Higher % SHL was observed in the sucrose group compared with NaCl and starch ($p < 0.05$), and no differences were detected between starch and NaCl groups ($p > 0.05$).

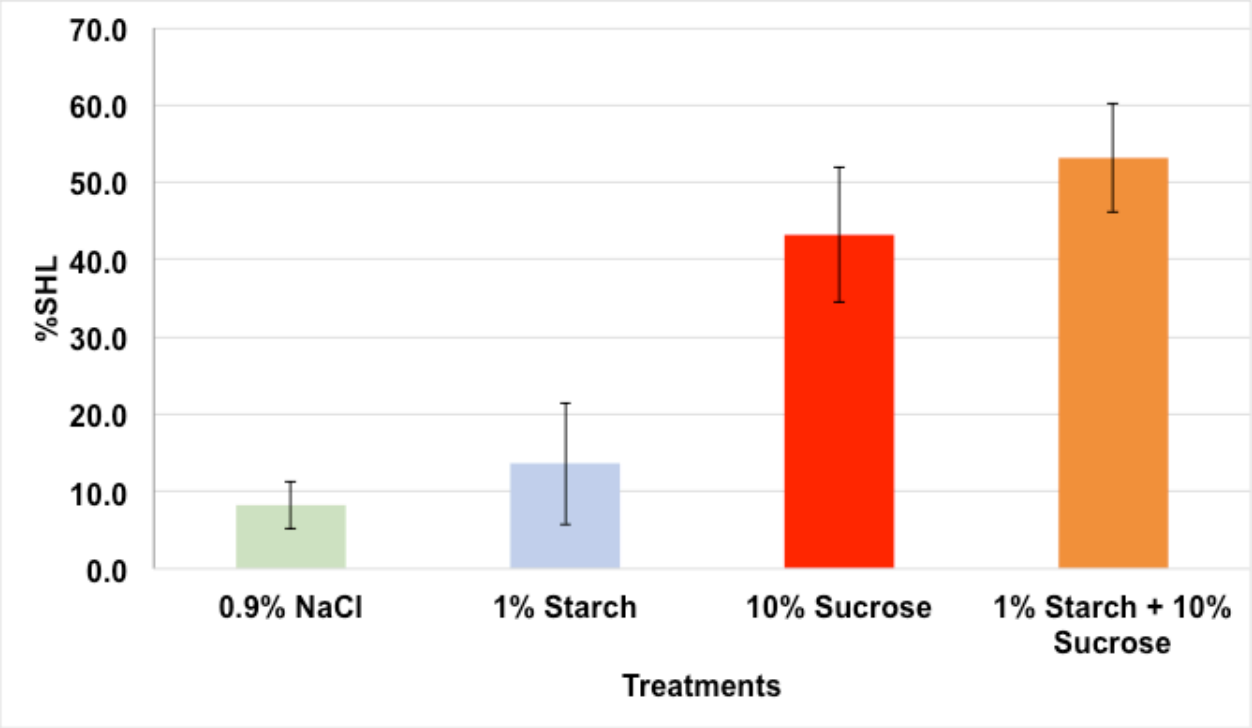

Figure 3. Percentage of surface hardness loss (%SHL) on dentine according to the treatments (mean ± SD, n = 18). Distinct letters indicate significant differences among the treatments ($p < 0.05$).

Discussion

The aim of the present study was to give support to previous publications, which have suggested that starch could increases the cariogenic potential of sucrose, thereby increasing dentine demineralization compared with sucrose alone. We used a biofilm model containing bacteria involved in starch metabolism [Cavacanti et al., 2014]. In addition, we contemplated the effect of salivary amylase on starch during the treatments [Botelho et al., 2016]. We believe that our approach clarifies the controversial findings about the effect of starch on the cariogenicity of sucrose reported in the literature [Ribeiro et al., 2005; Thurnheer et al., 2008; Aires et al., 2008; Duarte et al., 2008; Klein et al., 2009; Xiao and Koo, 2010; Botelho et al., 2016].

Our findings clearly showed (Figure 3) that the biofilm exposed to starch+sucrose provoked greater demineralization ($p < 0.05$) on dentine in comparison with the effect of sucrose alone. This result is supported by the lower pH ($p < 0.05$) found in the culture media after daily treatments with the starch+sucrose combination compared to sucrose (Figure 1). This lower pH was not caused by differences in biomass, total viable bacteria, or amount of polysaccharides in biofilms exposed to the starch+sucrose combination compared with sucrose (Table 1).

Indeed, the biofilm formed under exposure to starch+sucrose combination did not differ ($p > 0.05$) from sucrose in terms of biomass and viable bacteria. In addition, the results also may not be attributed to the fermentation of stored intracellular polysaccharides, since the amount of IPS did not differ between biofilms exposed to starch+sucrose or to sucrose alone. Furthermore, the findings also may not be consequence of the amount of extracellular polysaccharides found because the biofilms formed under starch+sucrose exposure did not differ from sucrose in terms of SEPS and IEPS. Moreover, the results may not be attributed to the earlier biofilm organization and maturation under combination of starch+sucrose, compared to sucrose-only group [Xiao and Koo, 2010], because statistically lower pH were observed since 32 h of biofilm growth and not only at the end of the study. Although we did not aim to explain why biofilm formed under starch+sucrose is more cariogenic than that exposed to sucrose alone, we have decided to extend this discussion aiming future researches.

In our cariogenic biofilm model [Ccahuana-Vásquez and Cury, 2010], biofilms are exposed eight times/day to the treatment solutions. At every specified time, the biofilms are immersed in the solutions during 3 min, washed and transferred to the culture media. During this 3 min carbohydrates should penetrate the biofilm matrix, reach the bacteria, and be fermented into acids. The acids produced are secreted by the bacteria into the biofilm fluid and diffuse into the culture media. Therefore, decreasing medium pH may be explained by an increased diffusion rate of the carbohydrates into the biofilm matrix and/or by retention of acids for longer in the biofilm matrix before being released into the culture media.

Therefore, the reduced pH and increased demineralization of dentine induced by starch and sucrose combination may be explained by two mechanisms: (*i*) starch combined with sucrose induces unique changes in the structure of the biofilm matrix, which increases the diffusion of sugars throughout the biofilm; or (*ii)* sucrose alters the structure of the biofilm, allowing starch to diffuse through the biofilm and ferment. The first hypothesis has been defended because starch

changes the chemical structure of EPS formed in the biofilm from sucrose [Bowen and Koo, 2011]. However, the cause-effect of this association has not been clearly determined. The second hypothesis used to explain why a biofilm exposed to starch+sucrose is more cariogenic than that exposed to sucrose alone would not be the complex effect of starch on biofilm structure, but the very well know effect of sucrose increasing the porosity of biofilm formed [Dibdin and Shellis, 1988]. Thus, starch is not easily fermented by biofilm due to its limited diffusion [Thurnheer et al., 2003], but diffusion is increased if the biofilm is formed under sucrose exposure. Therefore, starch can have cariogenic effects in the presence of sucrose simply because it can be fermented by the biofilm. Our findings support this hypothesis because the pH did not fall when the biofilm was exposed only to starch (Figure 1). This explanation is also supported by data of greater starch fermentation by dental plaques formed in human subjects during periods of dietary sucrose supplementation than limitation [Dodds and Edgar, 1986].

Sucrose changes the porosity of the biofilm matrix, therefore increasing the diffusion of high molecular weight sugars into the matrix. In addition, acids produced during sugar exposure are retained for longer on the biofilm matrix before diffusing through [Xiao and Koo, 2010]. However, whether more acids are retained by biofilm matrices exposed to starch and sucrose combination and whether this leads to increased dental demineralization remains to be elucidated.

A possible limitation of the present study was that the carbohydrate concentration of the starch+sucrose solution (11%) was higher than the sucrose solution (10%). We decided to use the same concentrations used in previous experiments [Botelho et al., 2016] to allow a direct comparison of the results. In addition, 1% starch + 10% sucrose does not cause higher demineralization on dentine and enamel than 1% starch + 9% sucrose [Botelho et al., 2016]. Another limitation was that the sucrose concentration (10%) was 10-fold the concentration of starch (1%). Sucrose is a disaccharide and starch is a polysaccharide, therefore these molecules differ in the moles of fermentable moieties. However, we tested this possibility. We fixed the starch concentration at 1% and varied the sucrose concentration from 2.5% to 10%. We found that demineralization was proportional to the concentration of sucrose in absence or presence of starch (data not shown).

In summary, using a suitable biofilm model to evaluate the cariogenicity of dietary starchy products we have showed additional evidences that starch increases the cariogenic

potential of sucrose but further studies should be conducted to elucidate the mechanism involved in pH reduction and greater demineralization found.

Acknowledgements

This study was supported by Conselho Nacional de Pesquisa e Desenvolvimento Científico e Tecnológico CNPQ (475178/2011 and 305310/2011 to Jaime Aparecido Cury). The first author received a scholarship from Capes, during her graduate course in Piracicaba Dental School, UNICAMP. The authors would like to thank the volunteers who provided human saliva for this experiment.

Author Contributions

Conceived and designed the experiment: J.A.C, S.E.S., A.A.D.B.C., Y.W.C; performed the experiment: S.E.S, A.A.S, A.P.R.F; analyzed the data: S.E.S, Y.W.C., J.A.C; wrote the draft manuscript: S.E.S, J.A.C; reviewed and approved the final manuscript: all authors.

References

Aires CP, Del Bel Cury AA, Tenuta LMA, Klein MI, Koo H, Duarte S, Cury JA: Effect of starch and sucrose on dental biofilm formation and on root dentine demineralization. Caries Res 2008;42:380-386.

Birkhed D, Skude G: Relation of amylase to starch and Lycasin® metabolism in human dental plaque in vitro. Scand J Dent Res 1978;86:248-258.

Botelho J, Villegas-Salinas M, Troncoso-Gajardo P, Giacaman R, Cury J: Enamel and dentine demineralization by a combination of starch and sucrose in a biofilm–caries model. Braz Oral Res 2016;30:1-8.

Bowen WH, Koo H: Biology of *Streptococcus mutans*-derived glucosyltransferases: Role in extracellular matrix formation of cariogenic biofilms. Caries Res 2011;45:69-86.

Cavalcanti YW, Bertolini MM, da Silva WJ, Del Bel Cury AA, Tenuta LMA, Cury JA: A three-species biofilm model for the evaluation of enamel and dentin demineralization. Biofouling 2014;30:579-588.

Ccahuana-Vásquez RA, Cury JA: *S. mutans* biofilm model to evaluate antimicrobial substances and enamel demineralization. Braz Oral Res 2010;24:135-141.

Cury JA, Rebelo MAB, Del Bel Cury AA, Derbyshire MTVC, Tabchoury CPM: Biochemical

composition and cariogenicity of dental plaque formed in the presence of sucrose or glucose and fructose. Caries Res 2000;34:491-497.

Dibdin GH, Shellis RP: Physical and biochemical studies of *Streptococcus mutans* sediments suggest new factors linking the cariogenicity of plaque with its extracellular polysaccharide content. J Dent Res 1988;67:890-895.

Dodds MW, Edgar, WM: Effects of dietary sucrose levels on pH fall and acid-anion profile in human dental plaque after a starch mouth-rinse. Archs Oral Biol 1986;31:509-512.

Duarte S, Klein MI, Aires CP, Cury JA, Bowen WH, Koo H: Influences of starch and sucrose on *Streptococcus mutans* biofilms. Oral Microbiol Immunol. 2008;23:206-212.

Fiehn NE, Moe D: Alpha-amylase activity in supragingival dental plaque in humans. Scand J Dent Res1983;91:365-370.

Glor EB, Miller CH, Spandau DF: Degradation of starch and its hydrolytic products by oral bacteria. J Dent Res 1988;67:75-81.

Hara AT, Queiroz CS, Paes Leme AF, Serra MC, Cury JA: Caries progression and inhibition in human and bovine root dentine in situ. Caries Res 2003;37:339-344.

Hartig SM: Basic image analysis and manipulation in ImageJ. Curr Protoc Mol Biol 2013; Chapter 14: Unit 14.15.

Jacobsen N, Melvaer KL, Hensten-Pettersen A: Some properties of salivary amylase. A survey of the literature and some observations. J Dent Res 1972;51:381-388.

Klein MI, Duarte S, Xiao J, Mitra S, Foster TH, Koo H: Structural and molecular basis of the role of starch and sucrose in *Streptococcus mutans* biofilm development. Appl Environ Microbiol 2009;75:837-841.

Koo H, Vacca-Smith AM, Bowen WH, Rosalen PL, Cury JA, Park YK: Effects of *Apis mellifera* propolis on the activities of streptococcal glucosyltransferases in solution and adsorbed onto saliva-coated hydroxyapatite. Caries Res 2000;34:418-426.

Lingström P, van Houte J, Kashket S: Food starches and dental caries. Crit Rev Oral Biol Med 2000;11:366-380.

Mormann JE, Muhlemann HR: Oral starch degradation and its influence on acid production in human dental plaque. Caries Res. 1981;15:166-175.

Nikitkova AE, Haase EM, Scannapieco FA: Taking the starch out of oral biofilm formation: molecular basis and functional significance of salivary α-amylase binding to oral streptococci.

Appl Environ Microbiol 2013;79:416-423.

Ribeiro CCC, Tabchoury CPM, Del Bel Cury AA, Tenuta LMA, Rosalen PL, Cury JA: Effect of starch on the cariogenic potential of sucrose. Br J Nutr 2005;94:44-50.

Sheiham A. Dietary effects on dental diseases. Public Health Nutr 2001;4:569-591.

Thurnheer T, Gmür R, Shapiro S, Guggenheim B: Mass transport of macromolecules within an in vitro model of supragingival plaque. Appl Environ Microbiol 2003;69:1702-1709.

Thurnheer T, Gierstsen E, Gmür R, Guggenheim B: Cariogenicity of soluble starch in oral in vitro biofilm and experimental rat caries studies: a comparison. Journal of Applied Microbiology 2008;105:829-836.

Vacca-Smith AM, Venkitaraman AR, Quivey RG, Bowen WH: Interactions of streptococcal glucosyltransferases with alpha-amylase and starch on the surface of saliva-coated hydroxyapatite. Arch Oral Biol 1996;41:291-298.

Vale GC, Tabchoury CPM, Del Bel Cury AA, Tenuta LMA, ten Cate JM, Cury JA: APF and dentifrice effect on root dentin demineralization and biofilm. J Dent Res 2011;90:77-81.

Xiao J, Koo H: Structural organization and dynamics of exopolysaccharide matrix and microcolonies formation by *Streptococcus mutans* in biofilms. J Appl Microbiol 2010;108:2103-2113.

3 CONCLUSÃO

Usando um modelo de biofilme mais apropriado para avaliar a cariogenicidade de produtos da dieta, os resultados obtidos mostraram associação amido + sacarose provoca maior desmineralização na dentina que o efeito isolado da sacarose.

A maior desmineralização encontrada foi coerente com a maior acidogenicidade do biofilme exposto a combinação de amido + sacarose e essa razão deve ser objeto de estudos futuros para explicar o mecanismo envolvido com o menor valor de pH encontrado.

REFERÊNCIAS*

Aires CP, Del Bel Cury AA, Tenuta LMA, Klein MI, Koo H, Duarte S, et al. Effect of starch and sucrose on dental biofilm formation and on root dentine demineralization. Caries Res. 2008;42(5):380–6.

Birkhed D, Skude G. Relation of amylase to starch and Lycasin® metabolism in human dental plaque in vitro. Scand J Dent Res. 1978;86:248-58.

Botelho JN, Villegas-Salinas M, Troncoso-Gajardo P, Giacaman RA, Cury JA. Enamel and dentine demineralization by a combination of starch and sucrose in a biofilm – caries model. Braz Oral Res. 2016;30(1):1–8.

Bowen WH, Koo H. Biology of *streptococcus mutans*-derived glucosyltransferases: Role in extracellular matrix formation of cariogenic biofilms. Caries Res. 2011;45(1):69–86.

Cavalcanti YW, Bertolini MM, da Silva WJ, Del-Bel-Cury AA, Tenuta LMA, Cury JA. A three-species biofilm model for the evaluation of enamel and dentin demineralization. Biofouling. 2014;30(5):579–88.

Ccahuana -Vásquez RA, Cury JA. *S. mutans* biofilm model to evaluate antimicrobial substances and enamel demineralization. Braz Oral Res. 2010;24(2):135–41.

Cury JA, Rebelo MAB, Del Bel Cury AA, Derbyshire MTVC, Tabchoury CPM. Biochemical composition and cariogenicity of dental plaque formed in the presence of sucrose or glucose and frutose. Caries Res. 2000;34:491–7.

* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committe of Medical Journal Editors – Vancouver Group. Abreviaturas dos periódicos em conformidade com o PubMed.

Dibdin GH, Shellis RP. Physical and biochemical studies of *Streptococcus mutans* sediments suggest new factors linking the cariogenicity of plaque with its extracellular polysaccharide content. J Dent Res. 1988;67:890–5.

Dodds MW, Edgar, WM. Effects of dietary sucrose levels on pH fall and acid-anion profile in human dental plaque after a starch mouth-rinse. Archs Oral Biol. 1986;31(8):509-12.

Duarte S, Klein MI, Aires CP, Cury JA, Bowen WH, Koo H. Influences of starch and sucrose on *Streptococcus mutans* biofilms. Oral Microbiol Immunol. 2008;23(3):206–12.

Fejerskov O. Changing paradigms in concepts on dental caries: consequences for oral health care. Caries Res. 2004;38(3):182–91.

Fiehn NE, Moe D. Alpha-amylase activity in supragingival dental plaque in humans. Scand J Dent Res.1983;91(5):365-70.

Giacaman RA, Munos MJ, Ccahuana-Vasquez RA, Munoz-Sandoval C, Cury JA. Effect of fluoridated milk on enamel and root dentin demineralization evaluated by a biofilm caries model. Caries Res 2012;46:460–6.

Glor EB, Miller CH, Spandau DF. Degradation of starch and its hydrolytic products by oral bacteria. J Dent Res. 1988;67(1):75-81.

Gupta B, Marya C, Juneja V, Dahiya V. Root Caries : An Aging Problem. 2006;1–6.

Hoppenbrouwers PM, Driessens FC, Borggreven JM. The vulnerability of unexposed human

dental roots to remineralization. J Dent Res. 1986;65:955-8.

Jacobsen N, Melvaer KL, Hensten-Pettersen A. Some properties of salivary amylase. A survey of the literature and some observations. J Dent Res. 1972;51:381-8.

Klein MI, Duarte S, Xiao J, Mitra S, Foster TH, Koo H. Structural and molecular basis of the role of starch and sucrose in *Streptococcus mutans* biofilm development. Appl Environ Microbiol. 2009;75(3):837–41.

Lingström P, Birkhed D, Ruben J, Arends J. Effect of frequent consumption of starchy food items on enamel and dentin demineralization and on plaque pH in situ. J Dent Res. 1994;73:652-60.

McKenna G, Allen AF, Flynn A, O'Mahony D, DaMata C, Cronin M et al. Impact of tooth repleacemnet strategies on the nutrition status of partially-dentate elders. Gerodontology. 2012;29:883-90.

Mormann JE, Muhlemann HR. Oral starch degradation and its influence on acid production in human dental plaque. Caries Res. 1981;15:166-75.

Muñoz-Sandoval C, Muñoz-Cifuentes MJ, Giacaman RA, Ccahuana-Vasquez RA, Cury JA. Effect of bovine milk on *Streptococcus mutans* biofilm cariogenic properties and enamel and dentin demineralization. Pediatr Dent. 2012;34(7).

Nikitkova AE, Haase EM, Scannapieco FA. Taking the starch out of oral biofilm formation: molecular basis and functional significance of salivary α-amylase binding to oral streptococci. Appl Environ Microbiol. 2013;79(2):416–23.

Paes Leme AF, Koo H, Bellato CM, Bedi G, Cury JA. The role of sucrose in cariogenic dental biofilm formation – New insight. J Dent Res. 2006;85:878-87.

Ribeiro CCC, Tabchoury CPM, Del Bel Cury AA, Tenuta LMA, Rosalen PL, Cury J a. Effect of starch on the cariogenic potential of sucrose. Br J Nutr. 2005;94:44-50.

Rolla G, Scheie AA, Ciard JE. Role of sucrose in plaque formation. Scand J Dent Res. 1985; 93:105-11.

Saunders RH, Meyerowitz C. Dental caries in older adults. Dent Clin N Am. 2005;49:293–308.

Tenuta LM, Ricomini FIlho AP, Del Bel Cury AA, Cury JA. Effect of sucrose on the selection of mutans streptococci and lactobacilli in dental biofilm formed in situ. Caries Res 2006;40:546-9.

Tester RF, Qi X, Karkalas J. Hydrolysis of native starches with amylases. Anim Feed Sci Technol. 2006;130(1-2):39–54.

Thurnheer T, Gmür R, Shapiro S, Guggenheim B. Mass transport of macromolecules within an in vitro model of supragingival plaque. Appl Environ Microbiol. 2003;69(3):1702-9.

Thurnherr T, Gierstsen E, Gmur R, Guggenheim B. Cartiogenicity of soluble starch in oral in vitro biofilm and experimental rat caries studies: a comparison. Journal of Applied Microbiology. 2008;105:829-36.

Vacca-Smith AM, Venkitaraman AR, Quivey RG, Bowen WH. Interactions of streptococcal glucosyltransferases with alpha-amylase and starch on the surface of saliva-coated hydroxyapatite. Arch Oral Biol. 1996;41(3):291–8.

Wall Aw, Steele JG. The relationship between oral healthy and nutrition in older people. Mech Ageing De. 2004;125(12):853-7.

ANEXOS

ANEXO 1

Comprovante de submissão do artigo intitulado “Starch increases root dentine demineralization provoked by sucrose” no periódico científico Caries Research

Submission Confirmation

Print

Thank you for your submission

Submitted to
Caries Research

Manuscript ID
201701012

Title
STARCH INCREASES ROOT DENTINE DEMINERALIZATION PROVOKED BY SUCROSE

Authors
Souza, Samilly
Sampaio, Aline
Del Bel Cury, Altair
Cavalcanti, Yuri
Ricomini Filho, Antônio Pedro
Cury, Jaime

Date Submitted
22-Jan-2017

ANEXO 2

Certificado do Comité de Ética em Pesquisa da FOP-UNICAMP

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Efeito da associação de sacarose e amido na desmineralização do esmalte e dentina radicular, avaliada por modelos de biofilmes"**, protocolo nº 035/2012, dos pesquisadores Lívia Maria Andaló Tenuta, Aline Araújo Sampaio, Altair Antoninha Del Bel Cury, Jaime Aparecido Cury, Javier Ignacio Briones Rojas, Juliana Nunes Botelho, Martinna de Mendonça e Bertolini, Rayane Ramos Araujo, Samilly Evangelista Souza, Wander José da Silva e Yuri Wanderley Cavalcanti, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 05/07/2012, com alterações em 06/11/2013 e 06/11/2014.

The Ethics Committee in Research of the Piracicaba Dental School - University of Campinas, certify that the project **"Effect of combination of sucrose and starch on the demineralization of enamel and root dentin, evaluated by biofilm models"**, register number 035/2012, of Lívia Maria Andaló Tenuta, Aline Araújo Sampaio, Altair Antoninha Del Bel Cury, Jaime Aparecido Cury, Javier Ignacio Briones Rojas, Juliana Nunes Botelho, Martinna de Mendonça e Bertolini, Rayane Ramos Araujo, Samilly Evangelista Souza, Wander José da Silva and Yuri Wanderley Cavalcanti, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee on Jul 05, 2012; with alterations on Nov 06, 2013 and Nov 06, 2014.

Prof. Dr. Jacks Jorge Junior
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Felippe Bevilacqua Prado
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.